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### TRANSLATOR'S DECLARATION

I, Paul David Churchill Clarke, B.A., MITI., translator to Messrs. Taylor and Meyer of 20 Kingsmead Road, London, SW2 3JD, Great Britain, verify that I know well both the German and the English language, that I have prepared the attached English translation of 36 pages of the German Patent Application No 199 51 975.7 in the German language with the title:

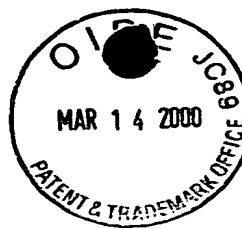
Neue für das poxB-Gen codierende Nukleotidsequenzen

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Date: 7 January 2000

By: P.D.C. Clarke



*B field of the invention*  
Novel nucleotide sequences coding for the poxB gene

## *BACKGROUND of the INvention*

The present invention provides nucleotide sequences from coryneform bacteria coding for the poxB gene and a process for the fermentative production of amino acids, in particular L-lysine, by attenuation of the poxB gene.

## *2. Background Information*

L-amino acids, in particular lysine, are used in human medicine and in the pharmaceuticals industry, in the food industry and very particularly in animal nutrition.

It is known that amino acids are produced by fermentation of strains of coryneform bacteria, in particular *Corynebacterium glutamicum*. Due to their great significance, efforts are constantly being made to improve the production process. Improvements to the process may relate to measures concerning fermentation technology, for example stirring and oxygen supply, or to the composition of the nutrient media, such as for example sugar concentration during fermentation, or to working up of the product by, for example, ion exchange chromatography, or to the intrinsic performance characteristics of the microorganism itself.

The performance characteristics of these microorganisms are improved using methods of mutagenesis, selection and mutant selection. In this manner, strains are obtained which are resistant to antimetabolites or are auxotrophic for regulatorily significant amino acids and produce amino acids.

For some years, the methods of recombinant DNA technology have also been used for strain improvement of strains of *Corynebacterium* which produce L-amino acid.

*C*  
SUMMARY OF THE INVENTION

Object of the invention

The inventors set themselves the object of providing novel measures for the improved fermentative production of amino acids, in particular L-lysine.

## Description of the invention

L-amino acids, in particular lysine, are used in human medicine and in the pharmaceuticals industry, in the food industry and very particularly in animal nutrition. There  
5 is accordingly general interest in providing novel improved process for the production of amino acids, in particular L-lysine.

The present invention provides an isolated polynucleotide containing a polynucleotide sequence selected from the  
10 group

- a) polynucleotide which is at least 70% identical to a polynucleotide which codes for a polypeptide containing the amino acid sequence of SEQ ID no. 2,
- b) polynucleotide which codes for a polypeptide which  
15 contains an amino acid sequence which is at least 70% identical to the amino acid sequence of SEQ ID no. 2,
- c) polynucleotide which is complementary to the polynucleotides of a) or b) and
- d) polynucleotide containing at least 15 successive bases  
20 of the polynucleotide sequence of a), b) or c).

The present invention also provides the polynucleotide as claimed in claim 1, wherein it preferably comprises a replicable DNA containing:

- (i) the nucleotide sequence shown in SEQ ID no. 1, or
- 25 (ii) at least one sequence which matches the sequence(i) within the degeneration range of the genetic code, or
- (iii) at least one sequence which hybridises with the complementary sequence to sequence (i) or (ii)  
30 and optionally

(iv) functionally neutral sense mutations in (i).

The present invention also provides

a polynucleotide according to claim 2, containing the nucleotide sequence as shown in SEQ ID no. 1,

5 a polynucleotide as claimed in claim 2 which codes for a polypeptide which contains the amino acid sequence as shown in SEQ ID no. 2,

a vector containing the polynucleotide as claimed in claim 1, point d, in particular pCR2.1poxBint, deposited in  
10 E. coli DSM 13114

and coryneform bacteria acting as host cell which contain an insertion or deletion in the pox gene.

The present invention also provides polynucleotides which substantially consist of a polynucleotide sequence, which  
15 are obtainable by screening by means of hybridisation of a suitable gene library, which contains the complete gene having the polynucleotide sequence according to SEQ ID no. 1, with a probe which contains the sequence of the stated polynucleotide according to SEQ ID no. 1 or a fragment  
20 thereof and isolation of the stated DNA sequence.

Polynucleotide sequences according to the invention are suitable as hybridisation probes for RNA, cDNA and DNA in order to isolate full length cDNA which code for the lrp protein and to isolate such cDNA or genes, the sequence of  
25 which exhibits a high level of similarity with that of the pyruvate oxidase gene.

Polynucleotide sequences according to the invention are furthermore suitable as primers for the production of DNA of genes which code for pyruvate oxidase by the polymerase  
30 chain reaction (PCR).

Such oligonucleotides acting as probes or primers contain at least 30, preferably at least 20, very particularly preferably at least 15 successive nucleotides.

5 Oligonucleotides having a length of at least 40 or 50 bases are also suitable.

"Isolated" means separated from its natural surroundings.

"Polynucleotide" generally denotes polyribonucleotides and polydeoxyribonucleotides, wherein the RNA or DNA may be unmodified or modified.

10 "Polypeptides" is taken to mean peptides or proteins which contain two or more amino acids joined via peptide bonds.

The polypeptides according to the invention include a polypeptide according to SEQ ID no. 2, in particular those having the biological activity of pyruvate oxidase and also  
15 those which are at least 70%, preferably at least 80% and in particular 90% to 95% identical to the polypeptide according to SEQ ID no. 2 and exhibit the stated activity.

The invention furthermore relates to a process for the fermentative production of amino acids, in particular  
20 lysine, using coryneform bacteria, which in particular already produce the amino acids, in particular L-lysine, and in which the nucleotide sequences which code for the poxB gene are attenuated, in particular are expressed at a low level.

25 In this connection, the term "attenuation" means reducing or suppressing the intracellular activity of one or more enzymes (proteins) in a microorganism, which enzymes are coded by the corresponding DNA, for example by using a weak promoter or a gene or allele which codes for a  
30 corresponding enzyme which has a low activity or inactivates the corresponding gene or enzyme (protein) and optionally by combining these measures.

- The microorganisms, provided by the present invention, may produce amino acids, in particular lysine, from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. The microorganisms
- 5 may comprise representatives of the coryneform bacteria in particular of the genus *Corynebacterium*. Within the genus *Corynebacterium*, *Corynebacterium glutamicum* may in particular be mentioned, which is known in specialist circles for its ability to produce L-amino acids.
- 10 Suitable strains of the genus *Corynebacterium*, in particular of the species *Corynebacterium glutamicum*, are in particular the known wild type strains
- Corynebacterium glutamicum* ATCC13032  
*Corynebacterium acetoglutamicum* ATCC15806  
15 *Corynebacterium acetoacidophilum* ATCC13870  
*Corynebacterium melassecola* ATCC17965  
*Corynebacterium thermoaminogenes* FERM BP-1539  
*Brevibacterium flavum* ATCC14067  
*Brevibacterium lactofermentum* ATCC13869 and  
20 *Brevibacterium divaricatum* ATCC14020
- and amino acid producing mutants or strains produced therefrom, such as for example
- such as for example the L-lysine producing strains
- Corynebacterium glutamicum* FERM-P 1709  
25 *Brevibacterium flavum* FERM-P 1708  
*Brevibacterium lactofermentum* FERM-P 1712  
*Corynebacterium glutamicum* FERM-P 6463  
*Corynebacterium glutamicum* FERM-P 6464 and  
*Corynebacterium glutamicum* DSM5714
- 30 The inventors succeeded in isolating the novel *poxB* gene, which codes for the enzyme pyruvate oxidase (EC 1.2.2.2), from *C. glutamicum*.

The *poxB* gene or also other genes are isolated from *C. glutamicum* by initially constructing a gene library of this microorganism in *E. coli*. The construction of gene libraries is described in generally known textbooks and  
5 manuals. Examples which may be mentioned are the textbook by Winnacker, *Gene und Klone, Eine Einführung in die Gentechnologie* (Verlag Chemie, Weinheim, Germany, 1990) or the manual by Sambrook et al.: *Molecular Cloning, A Laboratory Manual* (Cold Spring Harbor Laboratory Press,  
10 1989). One very well known gene library is that of *E. coli* K-12 strain W3110, which was constructed by Kohara et al. (Cell 50, 495-508 (1987)) in  $\lambda$ -vectors. Bathe et al. (Molecular and General Genetics, 252:255-265, 1996) describe a gene library of *C. glutamicum* ATCC13032, which  
15 was constructed using the cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA, 84:2160-2164) in *E. coli* K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575). Börmann et al. (Molecular Microbiology 6(3), 317-326, 1992) also  
20 describe a gene library of *C. glutamicum* ATCC13032, using cosmid pH79 (Hohn and Collins, Gene 11, 291-298 (1980)). O'Donohue (The Cloning and Molecular Analysis of Four Common Aromatic Amino Acid Biosynthetic Genes from *Corynebacterium glutamicum*. Ph.D. Thesis, National  
25 University of Ireland, Galway, 1997) describes the cloning of *C. glutamicum* genes using the  $\lambda$  Zap Expression system described by Short et al. (Nucleic Acids Research, 16: 7583).

A gene library of *C. glutamicum* in *E. coli* may also be  
30 produced using plasmids such as pBR322 (Bolivar, Life Sciences, 25, 807-818 (1979)) or pUC9 (Vieira et al., 1982, Gene, 19:259-268). Suitable hosts are in particular those *E. coli* strains with restriction and recombination defects, such as for example strain DH5 $\alpha$  (Jeffrey H. Miller: "A  
35 Short Course in Bacterial Genetics, A Laboratory Manual and



Handbook for Escherichia coli and Related Bacteria", Cold Spring Harbor Laboratory Press, 1992).

The long DNA fragments cloned with the assistance of cosmids or other  $\lambda$  vectors may then in turn be sub-cloned  
5 in conventional vectors suitable for DNA sequencing.

DNA sequencing methods are described, inter alia, in Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America USA, 74:5463-5467, 1977).

The resultant DNA sequences may then be investigated using  
10 known algorithms or sequence analysis programs, for example Staden's program (Nucleic Acids Research 14, 217-232(1986)), Butler's GCG program (Methods of Biochemical Analysis 39, 74-97 (1998)), Pearson & Lipman's FASTA algorithm (Proceedings of the National Academy of Sciences  
15 USA 85,2444-2448 (1988)) or Altschul et al.'s BLAST algorithm (Nature Genetics 6, 119-129 (1994)) and compared with the sequence entries available in publicly accessible databases. Publicly accessible nucleotide sequence  
databases are, for example, the European Molecular  
20 ~~Biology Laboratory~~ <sup>^</sup> Biologies Laboratories database ~~(sic)~~ (EMBL, Heidelberg, Germany) or the National Center for Biotechnology Information database (NCBI, Bethesda, MD, USA).

The novel DNA sequence from C. glutamicum which codes for the poxB gene and, as SEQ ID no. 1, is provided by the  
25 present invention, was obtained in this manner. The amino acid sequence of the corresponding protein was furthermore deduced from the above DNA sequence using the methods described above. The resultant amino acid sequence of the poxB gene product is shown in SEQ ID no. 2.

30 Coding DNA sequences arising from SEQ ID no. 1 due to the degeneracy of the genetic code are also provided by the present invention. DNA sequences which hybridise with SEQ ID no. 1 or parts of SEQ ID no. 1 are similarly provided by

the invention. Finally, DNA sequences produced by the polymerase chain reaction (PCR) using primers obtained from SEQ ID no. 1 are also provided by the present invention.

5 The person skilled in the art may find instructions for identifying DNA sequences by means of hybridisation inter alia in the manual "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology (1991) 41: 255-260). The person  
10 skilled in the art will find instructions for amplifying DNA sequences by means of the polymerase chain reaction (PCR) inter alia in the textbook by Gait, Oligonucleotide synthesis: a practical approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham, PCR (Spektrum Akademischer  
15 Verlag, Heidelberg, Germany, 1994).

The inventors discovered that coryneform bacteria produce L-amino acids, in particular L-lysine, in an improved manner once the *poxB* has been attenuated.

20 Attenuation may be achieved by reducing or suppressing either expression of the *poxB* gene or the catalytic properties of the enzyme protein. These measures may optionally be combined.

Reduced gene expression may be achieved by appropriate control of the culture or by genetic modification  
25 (mutation) of the signal structures for gene expression. Signal structures for gene expression are, for example, repressor genes, activator genes, operators, promoters, attenuators, ribosome binding sites, the start codon and terminators. The person skilled in the art will find  
30 information in this connection for example in patent application WO 96/15246, in Boyd & Murphy (Journal of Bacteriology 170: 5949 (1988)), in Voskuil & Chambliss (Nucleic Acids Research 26: 3548 (1998)), in Jensen & Hammer (Biotechnology and Bioengineering 58: 191 (1998)),

in Patek et al. (Microbiology 142: 1297 (1996)) and in known textbooks of genetics and molecular biology, such as for example the textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990).

Mutations which give rise to a change or reduction in the catalytic properties of enzyme proteins are known from the prior art; examples which may be mentioned are the papers by Qiu and Goodman (Journal of Biological Chemistry 272: 8611-8617 (1997)), Sugimoto et al. (Bioscience Biotechnology and Biochemistry 61: 1760-1762 (1997)) and Möckel ("Die Threonindehydratase aus Corynebacterium glutamicum: Aufhebung der allosterischen Regulation und Struktur des Enzyms", Berichte des Forschungszentrums Jülichs, Jül-2906, ISSN09442952, Jülich, Germany, 1994). Summary explanations may be found in known textbooks of genetics and molecular biology, such as for example that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

Mutations which may be considered are transitions, transversions, insertions and deletions. Depending upon the effect of exchanging the amino acids upon enzyme activity, the mutations are known as missense mutations or nonsense mutations. Insertions or deletions of at least one base pair in a gene give rise to frame shift mutations, as a result of which the incorrect amino acids are inserted or translation terminates prematurely. Deletions of two or more codons typically result in a complete breakdown of enzyme activity. Instructions for producing such mutations belong to the prior art and may be found in known textbooks of genetics and molecular biology, such as for example the textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft,

Weinheim, Germany, 1990) or by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

One example of a plasmid with the assistance of which insertion mutagenesis of the *poxB* gene may be performed is  
5 pCR2.1poxBint (Figure 1).

Plasmid pCR2.1poxBint consists of the plasmid pCR2.1-TOPO described by Mead et al. (Bio/Technology 9:657-663 (1991)), into which an internal fragment of the *poxB* gene, shown in SEQ ID no. 3, has been incorporated. After transformation  
10 and homologous recombination into the chromosomal *poxB* gene (insertion), this plasmid results in a total loss of enzyme function. By way of example, the strain DSM5715::pCR2.1poxBint, the pyruvate oxidase of which is switched off, was produced in this manner. Further  
15 instructions and explanations relating to insertion mutagenesis may be found, for example, in Schwarzer and Pühler (Bio/Technology 9,84-87 (1991)) or Fitzpatrick et al. (Applied Microbiology and Biotechnology 42, 575-580 (1994)).

20 It may additionally be advantageous for the production of L-amino acids, in particular L-lysine, in addition to attenuating the *poxB* gene, to amplify, in particular to overexpress, one or more enzymes of the particular biosynthetic pathway, of glycolysis, of anaplerotic  
25 metabolism, of the citric acid cycle or of amino acid export.

Thus, for example, for the production of L-lysine

- the *dapA* gene (EP-B 0 197 335) which codes for dihydropicolinate synthase may simultaneously be  
30 overexpressed, or
- the *dapD* gene (Wehrmann et al., Journal of Bacteriology 180, 3159-3165 (1998)) which codes for

tetradihdropicolinate succinylase may simultaneously be overexpressed, or

- the dapE gene (Wehrmann et al., Journal of Bacteriology 177: 5991-5993 (1995)) which codes for  
5 succinyldiaminopimelate desuccinylase may simultaneously be overexpressed, or
- the gap gene (Eikmanns (1992), Journal of Bacteriology 174:6076-6086) which codes for glyceraldehyde 3-phosphate dehydrogenase may simultaneously be overexpressed, or
- 10 • the pyc gene (Eikmanns (1992), Journal of Bacteriology 174:6076-6086) which codes for pyruvate carboxylase may simultaneously be overexpressed, or
- the mqo gene (Molenaar et al., European Journal of Biochemistry 254, 395 - 403 (1998)) which codes for  
15 malate:quinone oxidoreductase may simultaneously be overexpressed, or
- the lysE gene (DE-A-195 48 222) which codes for lysine export may simultaneously be overexpressed.

It may furthermore be advantageous for the production of  
20 amino acids, in particular L-lysine, in addition to attenuating the poxB gene, to suppress unwanted secondary reactions (Nakayama: "Breeding of Amino Acid Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London,  
25 UK, 1982).

The microorganisms containing the polynucleotide according to claim 1 are also provided by the invention and may be cultured continuously or discontinuously using the batch process or the fed batch process or repeated fed batch  
30 process for the purpose of producing L-amino acids, in particular L-lysine. A summary of known culture methods is given in the textbook by Chmiel (Bioprozesstechnik 1.

Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

- 5 The culture medium to be used must adequately satisfy the requirements of the particular strains. Culture media for various microorganisms are described in "Manual of Methods for General Bacteriology" from the American Society for Bacteriology (Washington D.C., USA, 1981). Carbon sources  
10 which may be used include sugars and carbohydrates, such as for example glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as for example soya oil, sunflower oil, peanut oil and coconut oil, fatty acids, such as for example palmitic acid,  
15 stearic acid and linoleic acid, alcohols, such as for example glycerol and ethanol, and organic acids, such as for example acetic acid. These substances may be used individually or as a mixture. Nitrogen sources which may be used comprise organic compounds containing nitrogen, such  
20 as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya flour and urea or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources may be used individually or  
25 as a mixture. Phosphorus sources which may be used are phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding salts containing sodium. The culture medium must furthermore contain metal salts, such as for example magnesium sulfate  
30 or iron sulfate, which are necessary for growth. Finally, essential growth-promoting substances such as amino acids and vitamins may also be used in addition to the above-stated substances. Suitable precursors may furthermore be added to the culture medium. The stated materials may be  
35 added to the culture in the form of a single batch or may be supplied in a suitable manner during culturing.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or ammonia water, or acidic compounds, such as phosphoric acid or sulfuric acid, are used appropriately to control the pH of the culture. Antifoaming agents, such as for example fatty acid polyglycol esters, may be used to control foaming. Suitable selectively acting substances, such as for example antibiotics, may be added to the medium in order to maintain plasmid stability. Oxygen or gas mixtures containing oxygen, such as for example air, are introduced into the culture in order to maintain aerobic conditions. The temperature of the culture is normally from 20°C to 45°C and preferably from 25°C to 40°C. The culture is continued until the maximum quantity of the desired amino acid has formed. This objective is normally achieved within 10 hours to 160 hours.

Methods for determining L-amino acids are known from the prior art. Analysis may proceed by anion exchange chromatography with subsequent ninhydrin derivatisation, as described in Spackman et al. (Analytical Chemistry, 30, (1958), 1190) or by reversed phase HPLC, as described in Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174).

The following microorganism has been deposited with Deutschen Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty:

- Escherichia coli strain DH5 $\alpha$ /pCR2.1poxBint as DSM 13114.

~~Examples~~ DETAILED DESCRIPTION OF THE INVENTION  
A

The present invention is illustrated in greater detail by the following practical examples.

Example 1

- 5 Production of a genomic cosmid gene library from  
Corynebacterium glutamicum ATCC13032

Chromosomal DNA from Corynebacterium glutamicum ATCC13032 was isolated as described in Tauch et al., (1995, Plasmid 33:168-179) and partially cleaved with the restriction  
10 enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, product description Sau3AI, code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, product description SAP, code no. 1758250). The  
15 DNA of cosmid vector SuperCos1 (Wahl et al. (1987) Proceedings of the National Academy of Sciences USA 84:2160-2164), purchased from Stratagene (La Jolla, USA, product description SuperCos1 Cosmid Vector Kit, code no. 251301) was cleaved with the restriction enzyme XbaI  
20 (Amersham Pharmacia, Freiburg, Germany, product description XbaI, Code no. 27-0948-02) and also dephosphorylated with shrimp alkaline phosphatase. The cosmid DNA was then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, product description BamHI,  
25 code no. 27-0868-04). Cosmid DNA treated in this manner was mixed with the treated ATCC 13032 DNA and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, product description T4 DNA Ligase, code no. 27-0870-04). The ligation mixture was then packed in phages  
30 using Gigapack II XL Packing Extracts (Stratagene, La Jolla, USA, product description Gigapack II XL Packing Extract, code no. 200217). E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Res. 16:1563-1575) was infected by suspending the cells in 10 mM MgSO<sub>4</sub> and mixing them with an



aliquot of the phage suspension. The cosmid library was infected and titred as described in Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), wherein the cells were plated out on LB agar  
5 (Lennox, 1955, Virology, 1:190) + 100µg/ml of ampicillin. After overnight incubation at 37°C, individual recombinant clones were selected.

## Example 2

### 10 Isolation and sequencing of the poxB gene

Cosmid DNA from an individual colony was isolated in accordance with the manufacturer's instructions using the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) and partially cleaved with the restriction  
15 enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, product description Sau3AI, code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, product description SAP, code no. 1758250). Once  
20 separated by gel electrophoresis, the cosmid fragments of a size of approx. 1500 to 2000 bp were isolated using the QiaExII Gel Extraction Kit (product no. 20021, Qiagen, Hilden, Germany). The DNA of the sequencing vector pZero-1 purchased from Invitrogen (Groningen, Netherlands, product  
25 description Zero Background Cloning Kit, product no. K2500-01) was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, product description BamHI, Product No. 27-0868-04). Ligation of the cosmid fragments into the sequencing vector pZero-1 was performed as  
30 described by Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), wherein the DNA mixture was incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then electroporated into the E. coli strain DH5aMCR (Grant,

1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649) (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) and plated out onto LB agar (Lennox, 1955, Virology, 1:190) + 50 µg/ml of Zeocin. Plasmids of  
5 the recombinant clones were prepared using the Biorobot 9600 (product no. 900200, Qiagen, Hilden, Germany, Germany). Sequencing was performed using the dideoxy chain termination method according to Sanger et al. (1977, Proceedings of the National Academies of Sciences U.S.A.,  
10 74:5463-5467) as modified by Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (product no. 403044, Weiterstadt, Germany) was used. Separation by gel electrophoresis and analysis of the  
15 sequencing reaction was performed in a "Rotiphorese NF" acrylamide/bisacrylamide gel (29:1) (product no. A124.1, Roth, Karlsruhe, Germany) using the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

20 The resultant raw sequence data were then processing using the Staden software package (1986, Nucleic Acids Research, 14:217-231), version 97-0. The individual sequences of the pZero 1 derivatives were assembled into a cohesive contig. Computer-aided coding range analysis was performed using  
25 XNIP software (Staden, 1986, Nucleic Acids Research, 14:217-231). Further analysis was performed using the "BLAST search programs" (Altschul et al., 1997, Nucleic Acids Research, 25:3389-3402), against the non-redundant database of the "National Center for Biotechnology  
30 Information" (NCBI, Bethesda, MD, USA).

The resultant nucleotide sequence is stated in SEQ ID no. 1 . Analysis of the nucleotide sequence revealed an open reading frame of 1737 base pairs, which was designated the poxB gene. The poxB gene codes for a  
35 polypeptide of 579 amino acids.

## Example 3

Production of an integration vector for integration  
mutagenesis of the poxB gene

Chromosomal DNA was isolated from strain ATCC 13032 using  
5 the method of Eikmanns et al. (Microbiology 140: 1817 -  
1828 (1994)). On the basis of the sequence of the poxB gene  
for *C. glutamicum* known from Example 2, the following  
oligonucleotides were selected for the polymerase chain  
reaction:

10 poxBint1:  
5' TGC GAG ATG GTG AAT GGT GG 3'  
poxBint2:  
5' GCA TGA GGC AAC GCA TTA GC 3'

The stated primers were synthesised by the company MWG  
15 Biotech (Ebersberg, Germany) and the PCR reaction performed  
in accordance with the standard PCR method of Innis et al.  
(PCR protocols. A guide to methods and applications, 1990,  
Academic Press) using Pwo polymerase from Boehringer. A DNA  
fragment of approx. 0.9 kb in size, which bears an internal  
20 fragment of the poxB gene and is shown in SEQ ID no. 3, was  
isolated with the assistance of the polymerase chain  
reaction.

The amplified DNA fragment was ligated into the vector  
pCR2.1-TOPO (Mead et al. (1991) Bio/Technology 9:657-663)  
25 using the TOPO TA Cloning Kit from Invitrogen Corporation  
(Carlsbad, CA, USA; catalogue no. K4500-01). The *E. coli*  
strain DH5 $\alpha$  was then electroporated with the ligation batch  
(Hanahan, in DNA cloning. A practical approach. Vol.I. IRL-  
Press, Oxford, Washington DC, USA, 1985). Plasmid-bearing  
30 cells were selected by plating the transformation batch out  
onto LB agar (Sambrook et al., Molecular cloning: a  
laboratory manual. 2<sup>nd</sup> Ed. Cold Spring Harbor Laboratory  
Press, Cold Spring Harbor, N.Y., 1989) which had been

DJS  
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supplemented with 25 mg/l of kanamycin. Plasmid DNA was isolated from a transformant using the QIAprep Spin Miniprep Kit from Qiagen and verified by restriction with the restriction enzyme EcoRI and subsequent agarose gel electrophoresis (0.8%). The plasmid was named pCR2.1poxBint.

#### Example 4

Integration mutagenesis of the poxB gene into the lysine producer DSM 5715

The vector named pCR2.1poxBint in Example 2 was electroporated into *Corynebacterium glutamicum* DSM 5715 using the electroporation method of Tauch et al. (FEMS Microbiological Letters, 123:343-347 (1994)). Strain DSM 5715 is an AEC-resistant lysine producer. The vector pCR2.1poxBint cannot independently replicate in DSM 5715 and is only retained in the cell if it has been integrated into the chromosome of DSM 5715. Clones with pCR2.1poxBint integrated into the chromosome were selected by plating the electroporation batch out onto LB agar (Sambrook et al., Molecular cloning: a laboratory manual. 2<sup>nd</sup> Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) which had been supplemented with 15 mg/l of kanamycin. Integration was detected by labelling the poxBint fragment with the Dig hybridisation kit from Boehringer using the method according to "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993). Chromosomal DNA of a potential integrant was isolated using the method according to Eikmanns et al. (Microbiology 140: 1817 - 1828 (1994)) and cut in each case with the restriction enzymes SalI, SacI and HindIII. The resultant fragments were separated by agarose gel electrophoresis and hybridised at 68°C using the Dig hybridisation kit from Boehringer. The plasmid named

pCR2.1poxBint in Example 3 had been inserted within the chromosomal poxB gene in the chromosome of DSM 5715. The strain was designated DSM5715::pCR2.1poxBint.

## 5 Example 5

### Production of lysine

The *C. glutamicum* strain DSM5715::pCR2.1poxBint obtained in Example 3 was cultured in a nutrient medium suitable for the production of lysine and the lysine content of the culture supernatant was determined.

To this end, the strain was initially incubated for 24 hours at 33°C on an agar plate with the appropriate antibiotic (brain/heart agar with kanamycin (25 mg/l)). Starting from this agar plate culture, a preculture was inoculated (10 ml of medium in a 100 ml Erlenmeyer flask). The complete medium CgIII was used as the medium for this preculture. Kanamycin (25 mg/l) was added to this medium. The preculture was incubated for 48 hours at 33°C on a shaker at 240 rpm. A main culture was inoculated from this preculture, such that the initial optical density (OD, 660 nm) of the main culture was 0.1 OD. Medium MM was used for the main culture.

## Medium MM

CSL (Corn Steep Liquor)	5 g/l
MOPS	20 g/l
Glucose (separately autoclaved)	50 g/l
Salts:	
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	25 g/l
KH <sub>2</sub> PO <sub>4</sub>	0.1 g/l
MgSO <sub>4</sub> * 7 H <sub>2</sub> O	1.0 g/l
CaCl <sub>2</sub> * 2 H <sub>2</sub> O	10 mg/l
FeSO <sub>4</sub> * 7 H <sub>2</sub> O	10 mg/l
MnSO <sub>4</sub> * H <sub>2</sub> O	5.0mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine*HCl (sterile-filtered)	0.2 mg/l
Leucine (sterile-filtered)	0.1 g/l
CaCO <sub>3</sub>	25 g/l

CSL, MOPS and the salt solution are adjusted to pH 7 with ammonia solution and autoclaved. The sterile substrate and  
5 vitamin solutions, together with the dry-autoclaved CaCO<sub>3</sub> are then added.

Culturing is performed in a volume of 10 ml in a 100 ml Erlenmeyer flask with flow spoilers. Kanamycin (25 ml/l) was added. Culturing was performed at 33°C and 80%  
10 atmospheric humidity.

## BRIEF DESCRIPTION OF THE DRAWING

- a
- After 48 hours, the OD was determined at a measurement wavelength of 660 nm using a Biomek 1000 (Beckmann Instruments GmbH, Munich). The quantity of lysine formed was determined using an amino acid analyser from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatisation with ninhydrin detection.

Table 1 shows the result of the test.

Table 1

Strain	OD(660)	Lysine HCl 5 g/l
DSM5715	13.1	9.5
DSM5715::pCR2.1poxBint	12.5	12.9

The following Figures are attached:

Figure 1: Map of the plasmid pCR2.1poxBint.

The abbreviations and terms used have the following meanings.

5

ColE1 ori:	Replication origin of the plasmid ColE1
lacZ:	5' end of the $\beta$ -galactosidase gene
f1 ori:	Replication origin of the f1 phage
KmR:	Kanamycin resistance
ApR:	Ampicillin resistance
BamHI:	Restriction site of the restriction enzyme BamHI
EcoRI:	Restriction site of the restriction enzyme EcoRI:
poxBint2:	Internal fragment of the poxB gene



## SEQUENCE LISTING

&lt;110&gt; Degussa-Hüls AG

5 &lt;120&gt; Novel nucleotide sequences coding for the poxB gene

&lt;130&gt; 990159 BT

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&lt;170&gt; PatentIn Ver. 2,1

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